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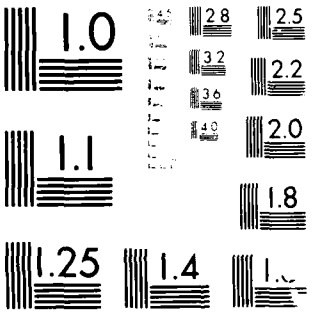

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The initial studies outlined in this report demonstrate that Biemann's approach to the sequencing of polypeptides by gas chromatography-mass spectrometry can be carried out on the bench top gas chromatograph-mass spectrometer - data system developed by Hewlett Packard. This technique has been used to determine the complete primary structure of glucagon and 50% of the primary structure of Tumor Necrosis Factor.

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Annual Report on Contract N00014-87-K-0016

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RESEARCH OBJECTIVE

The goal of this project is to determine if Biemann's approach to the sequencing of polypeptides by gas chromatography-mass spectrometry can be carried out on the bench top gas chromatograph-mass spectrometer-data system (GC-MS-DS) developed by Hewlett Packard (HP).

STUDIES COMPLETED DURING YEAR 1 (11/1/86-10/31/87)

Goal 1: To implement PEPALG and LSTGEN on the HP9816 microcomputer

PEPALG and LSTGEN, the algorithms required for the automated interpretation of the mass spectra of polyamino alcohols, were redesigned into modular form from FORTRAN source code supplied by Klaus Biemann and coded in PASCAL (revision 3.1). In addition, a large number of support procedures were implemented and integrated into a user interface that makes PEPALG an interactive program in the QUICKSILVER environment (the proprietary instrument control and data acquisition software developed by HP). These algorithms were then used to interpret the mass spectra obtained subsequently from glucagon and Tumor Necrosis Factor (TNF) [see goal 3 below].

Goal 2: To perform the studies on the HP5890A capillary GC and the HP5970B quadrupole MS necessary to determine the required constants used in PEPALG and LSTGEN

A. Determination of the GC conditions for implementation of the Kovats retention index (RI) scheme.

The GC conditions that would allow all of the components of a mixture of alkanes of the form C_nH_{2n+2} (n=12-44) to elute from the capillary column during a linear temperature program were determined from a large number of experiments performed with different stationary phases and different temperature programs. The use of these GC conditions make it possible to estimate the retention index of any unknown compound by linear interpolation between the RI's of hydrocarbons that differ by at most four carbon atoms. The GC conditions are as follows: HP 1 ULTRA capillary column (film thickness 0.33 μM, I.D. 0.20 mM, length 12 M); initial temperature 70°C, final temperature 310°C, rate 1.5°C/minute, final hold time 5 minutes. The total analysis time is 165 minutes.

B. Synthesis of model tripeptides for indexing the LSTGEN library

A series of nineteen tripeptides of the form Ala-X-Ala where X is one of twenty amino acids was prepared with a Biosearch Model 9500 peptide synthesizer; Ala-Ala-Ala was purchased from Sigma. The purities of the model tripeptides were determined by HPLC on a C18 column (Table 1).

Table 1

HPLC Properties of Ala-X-Ala

Peptide	Retention Time (min)	% Purity
Ala-Ala-Ala	7.57	92
Ala-Val-Ala	4.75	100
Ala-Leu-Ala	12.1	100
Ala-Ile-Ala	10.0	96
Ala-Pro-Ala	17.6	88
Ala-Phe-Ala	34.7	100
Ala-Trp-Ala	21.3	88
Ala-Met-Ala	6.47	88
Ala-Gly-Ala	6.91	87
Ala-Ser-Ala	6.87	91
Ala-Thr-Ala	8.37	83
Ala-Asn-Ala	6.39	79
Ala-Gln-Ala	7.78	82
Ala-Cys-Ala	18.9	100
Ala-Tyr-Ala	7.47	100
Ala-Asp-Ala	7.06	88
Ala-Glu-Ala	9.53	86
Ala-Lys-Ala	8.35	100
Ala-Arg-Ala	10.4	100
Ala-His-Ala	8.61	98

C. Implementation of the Chemistry for Synthesizing Polyamino Alcohol Derivatives of Peptides

Standard solutions (4.0 μg/mL) of model tripeptides were prepared in 0.005 M acetic acid that had been sparged with N_2. 200 μg (≈800 nmole) of the tripeptide were added to 4 mm PYREX test tubes that had been heated for 30 minutes. The samples were immediately placed in a PICO.TAG chamber and the acetic acid was removed under vacuum (60 mTorr).

The detailed procedures published by Biemann were used to implement the following sequence of chemical reactions:

1. Esterification with diazomethane
2. Acylation with methyl trifluoroacetate
3. Reduction with BD_3/THF
4. Silylation with pyridine/TMSDEA

Peptides containing arginine or cysteine undergo an additional derivatization step, outlined below, before they are submitted to the above sequence of reactions:

1. Hydrazinalysis with hydrazine hydrate
2. Carboxymethylation with iodoacetic acid.

The major changes that have been made in the derivatization chemistry are: 1) small reaction vessels (Pyrex 6x50 mm) have been used; 2) reactions have been scaled

down so μL quantities of reagents are used; 3) the reaction have been performed in the PICO.TAG workstation so that conditions can be reproduced exactly; 4) all reactions have been performed in an inert atmosphere (dry N_2); 5) all evaporation steps were performed with controlled vacuum.

D. GC-MS properties of the Derivatized Tripeptides

The polyamino alcohol derivatives of the model tripeptides were analyzed in the GC-MS system under the conditions that had been determined in our previous study of the hydrocarbon standard: transfer line 320°C; solvent delay 7.0 minutes; low mass 60; high mass 800; scans/second 1.17. The computed retention indices and the RI increment for each amino acid are contained in Table 2; the contribution of the constant portion of the tripeptides to the RI is 615. It should be pointed out that these RI increments are similar to those derived from the packed column work carried out by Biemann except that I(Leu) and I(Ile) are different because of the resolution of the capillary column used in these experiments; therefore, PEPALG can distinguish Leu and Ile.

Table 2

Retention Index Increments for Amino Acids

Peptide	RI	I(X)
Ala-Ala	1259	----------
Ala-Ala-Ala	1569	I(Ala) = 310
Ala-Val-Ala	1684	I(Val) = 425
Ala-Leu-Ala	1759	I(Leu) = 500
Ala-Ile-Ala	1764	I(Ile) = 505
Ala-Pro-Ala	1734	I(Pro) = 475
Ala-Phe-Ala	2094	I(Phe) = 835
Ala-Trp-Ala	2649	I(Trp) = 1390
Ala-Met-Ala	1999	I(Met) = 740
Ala-Gly-Ala	1589	I(Gly) = 330
Ala-Ser-Ala	1849	I(Ser) = 590
Ala-Thr-Ala	1824	I(Thr) = 565
Ala-Asn-Ala	1864	I(Asn) = 605
Ala-Gln-Ala	2069	I(Gln) = 810
Ala-Cys-Ala	2209	I(Cys) = 950
Ala-Tyr-Ala	2419	I(Tyr) = 1160
Ala-Asp-Ala	1939	I(Asp) = 680
Ala-Glu-Ala	2029	I(Glu) = 770
Ala-Lys-Ala	2089	I(Lys) = 830
Ala-Arg-Ala	1939	I(Arg) = 680
Ala-His-Ala	2374	I(His) = 1115

These parameters were then entered into LSTGEN which then generated a library of di- to penta-peptides (A-B-C-D-E) indexed by the number computed from the formula:

$$RI\ (A\text{-}B\text{-}C\text{-}D\text{-}E) = 615 + I(A) + I(B) + I(C) + I(D) + I(E).$$

The library was saved in a disc file.

Goal 3: To demonstrate that it is possible to determine the sequence of a test peptide and to estimate the overall sensitivity of this approach

Glucagon is a peptide of 29 amino acids that was used by Biemann as a test compound for his original experiments; the amount used for his experiments was between 400 and 1000 nmoles. The primary structure of glucagon with the amides converted to acids (as would occur under the conditions of an acid hydrolysis) is as follows:

1
His-Ser-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-
16
Ser-Arg-Arg-Ala-Glu-Asp-Phe-Val-Glu-Trp-Leu-Met-Asp-Thr

In order to test our derivatization procedures and software, 28.7 nmoles of glucagon was submitted to partial acid hydrolysis (6 N HCl at 120 °C for 1 hour) in an inert atmosphere of dry N_2; the resultant mixture of peptides was derivatized with the above procedures and 1/30th of the resulting material (approximately 1 nmole of hydrolysate) was analyzed by GC-MS. The total ion chromatogram (TIC), marked with the best matches identified by PEPALG, is shown in Figures 1-3. It is clear that the primary structure could be deduced from this experiment except for the Arg-Arg region; since the hydrolysate had not been subjected to hydrazinolysis, this gap is not unexpected. A second sample of glucagon was treated as above except that the hydrolysis was subjected to hydrazinolysis prior to the other derivatization reactions; the TIC, marked with the best matches identified by PEPALG, is shown in Figure 4 (Arg is converted to Orn in the hydrazinolysis reaction). It is clear that the structure of the Arg-Arg region is determined by this experiment.

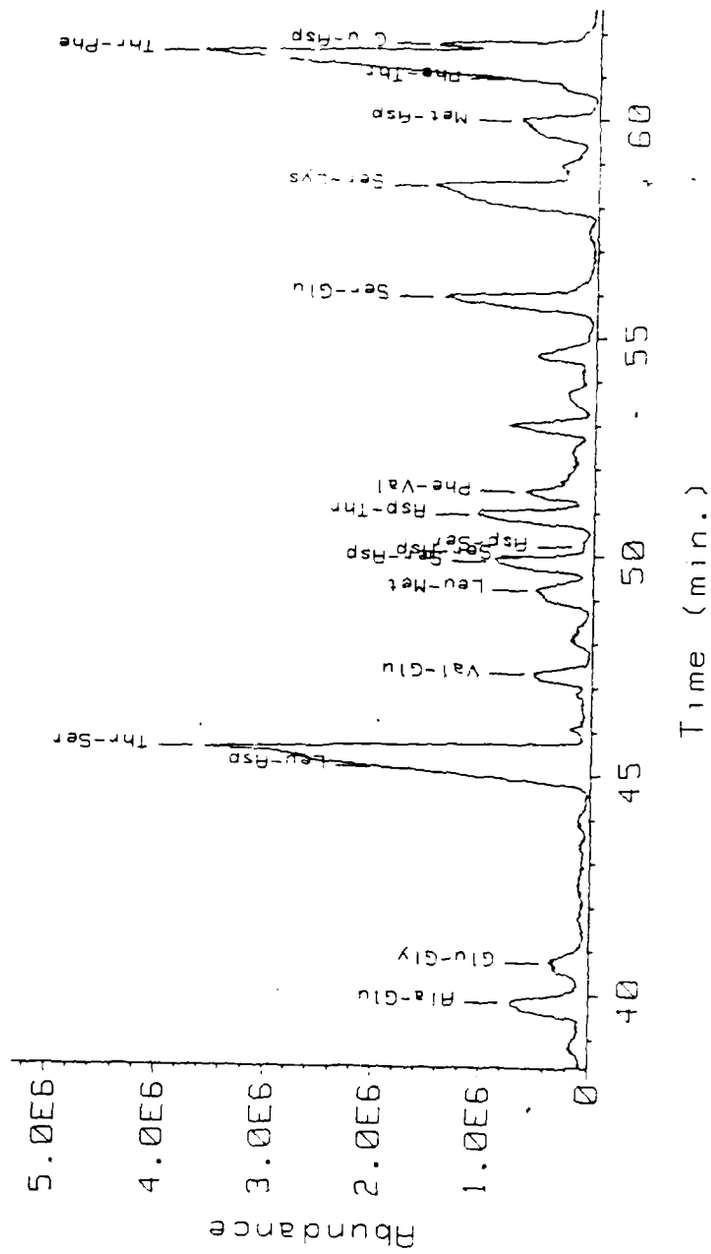
By examining the peptides identified in these two experiments, it is clear that there are sufficient overlaps to reconstruct the primary structure of the underlined portions of the glucagon molecule. It is especially interesting to note that the amount of material used has been reduced by at least a factor of 20 and that the primary structure of the C-terminus of the molecule was determined: Trp-Leu-Met-Asp-Thr.

Goal 4: To extend this technique to polypeptides of pharmacological interest in the range of 150-200 amino acids in length

TNF is a polypeptide of 155 amino acids that is secreted by macrophages and has cytotoxic activity. The primary structure of TNF with the amides converted to acids is as follows:

1
Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala-Asp-Pro-Glu-
20
Ala-Glu-Gly-Glu-Leu-Glu-Trp-Leu-Asp-Arg-Arg-Ala-Asp-Ala-Leu-Leu-Ala-Asp-Gly-
39
Val-Glu-Leu-Arg-Asp-Asp-Glu-Leu-Val-Val-Pro-Ser-Glu-Gly-Leu-Tyr-Leu-Ile-Tyr-
58
Ser-Glu-Val-Leu-Phe-Lys-Gly-Glu-Gly-Cys-Pro-Ser-Thr-His-Val-Leu-Leu-Thr-His-
77
Thr-Ile-Ser-Arg-Ile-Ala-Val-Ser-Tyr-Glu-Thr-Lys-Val-Asp-Leu-Leu-Ser-Ala-Ile-
96
Lys-Ser-Pro-Cys-Glu-Arg-Glu-Thr-Pro-Glu-Gly-Ala-Glu-Ala-Lys-Pro-Trp-Tyr-Glu-
115
Pro-Ile-Tyr-Leu-Gly-Gly-Val-Phe-Glu-Leu-Glu-Lys-Gly-Asp-Arg-Leu-Ser-Ala-Glu-
134
Ile-Asp-Arg-Pro-Asp-Tyr-Leu-Asp-Phe-Ala-Glu-Ser-Gly-Glu-Val-Tyr-Phe-Gly-Ile-
153
Ile-Ala-Leu

Figure 1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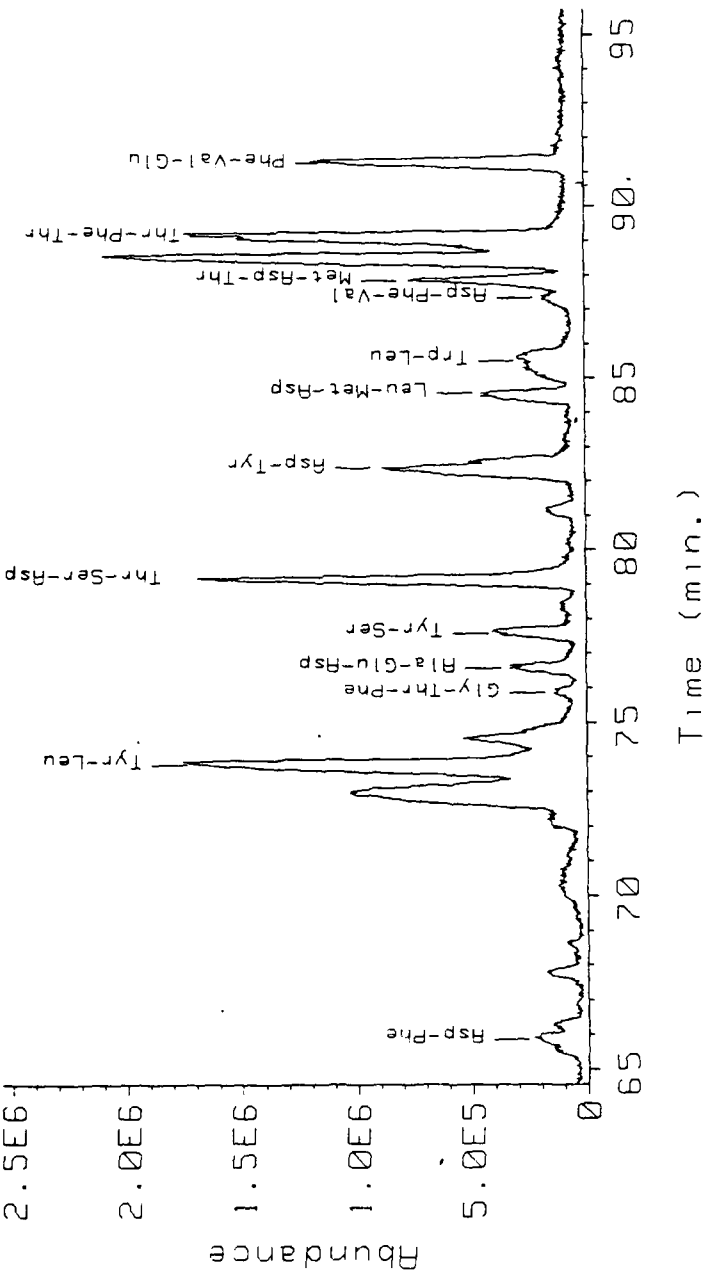
1.0E6
0
40
45
50
55
60
Time (min.)
Ala-Glu
Glu-Gly
Leu-Asp
Thr-Ser
Val-Glu
Leu-Met
Ser-Asp
Asp-Ser
Asp-Thr
Phe-Val
Ser-Glu
Ser-Lys
Met-Asp
Glu-Asp
Thr-Phe

Figure 2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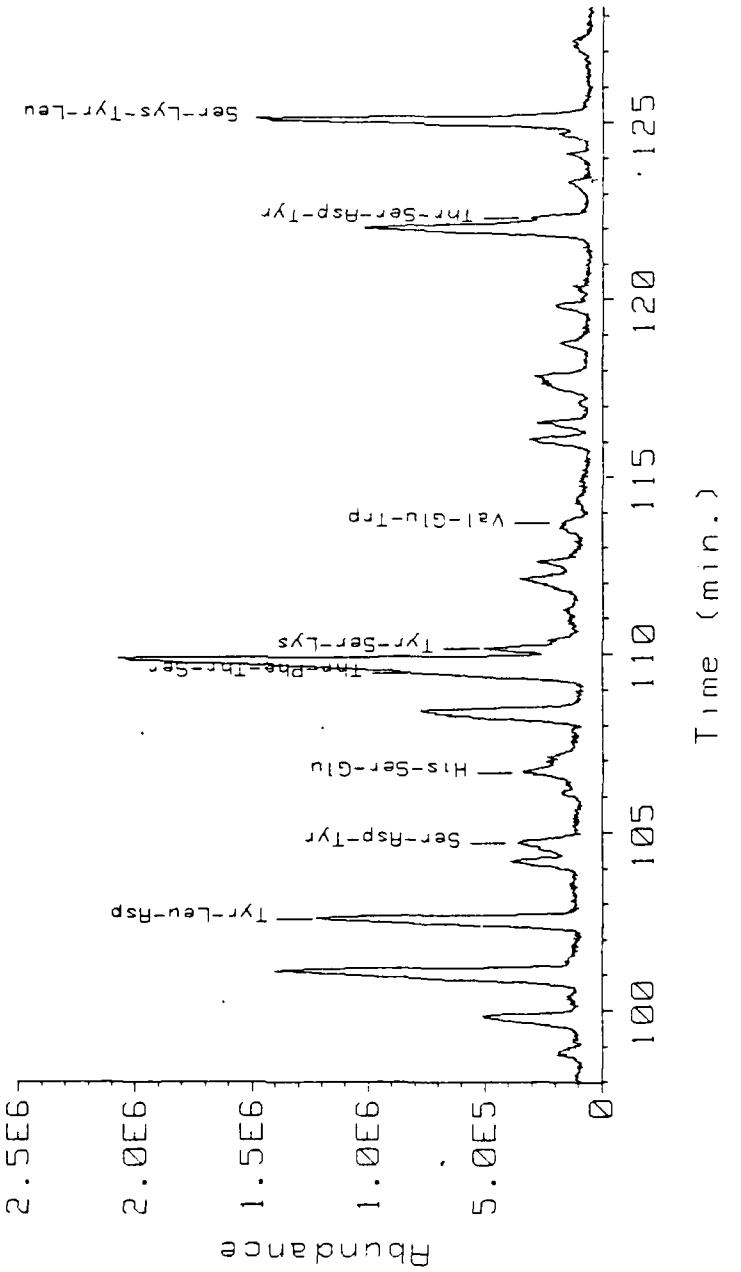
Time (min.)
Asp-Phe
Tyr-Leu
Gly-Thr-Phe
Ala-Glu-Asp
Tyr-Ser
Thr-Ser-Asp
Asp-Tyr
Leu-Met-Asp
Trp-Leu
Asp-Phe-Val
Met-Asp-Thr
Thr-Phe-Thr
Phe-Val-Glu

Figure 3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Ser-Asp-Tyr
His-Ser-Glu
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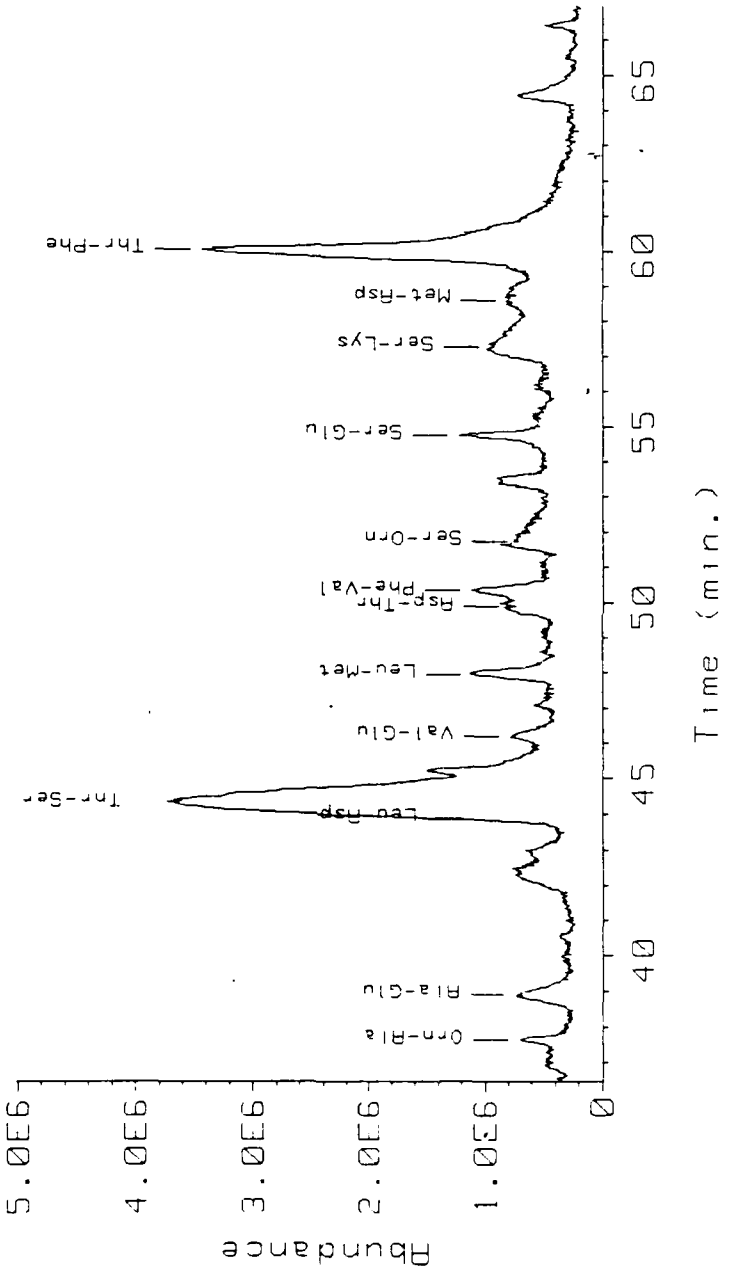
Val-Glu-Trp
Thr-Ser-Asp-Tyr
Ser-Lys-Tyr-Leu

Figure 4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Time (min.)
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45
50
55
60
65
Orn-Ala
Ala-Glu
Leu-Asp
Thr-Ser
Val-Glu
Leu-Met
Asp-Thr
Phe-Val
Ser-Orn
Ser-Glu
Ser-Lys
Met-Asp
Thr-Phe

30 nmoles of TNF was hydrolyzed, derivatized, and analyzed as described above for glucagon. In this exploratory experiment, 70 of the 155 amino acid residues were contained in the polypeptides identified (underlined areas of TNF primary structure).

STUDIES PLANNED FOR YEAR 2 (11/1/87-10/31/88)

Goal 1: To synthesize and purify oligopeptides of the form Ala-X-Ala-Ala and Ala-Ala-X-Ala-Ala where X is one of the twenty amino acids

Goal 2: To prepare the polyamino alcohol derivatives of these oligopeptides and study their GC and MS properties

Goal 3: To integrate the information obtained in Goal 2 into PEPALG

Goal 4: To determine a sequence of enzymatic reactions for preparing a more representative mixture of oligopeptides from TNF

Goal 5: To extend this technique to other polypeptides similar in size to TNF (human growth hormone, IL-1)

Goal 6: To initiate the study of the conformation of TNF in solution by high resolution NMR and molecular modeling and parallel structure/function studies

PUBLICATONS

A poster was presented at the first symposium of the Protien Society in August, 1987:

J. Wayne Cowens, Michael A. Reino, Larry H. Mead & M. Jane Ehrke. The Utility of a Benchtop Gas Chromatograph-Mass Spectrometer-Microcomputer Data System (GC-MS-DS) in Sequencing Polypeptides. Abstract # 812, The First Symposium of the Protein Society, San Diego, California, August 9-13, 1987.

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